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# Tissue Transglutaminase Facilitates the Polymerization of Insulin-like Growth Factor-binding Protein-1 (IGFBP-1) and Leads to Loss of IGFBP-1's Ability to Inhibit Insulin-like Growth Factor-I-stimulated Protein Synthesis*

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**Keiji Sakai, Walker H. Busby, Jr., Jane B. Clarke, and David R. Clemmons‡**

*From the Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599*

**Insulin-like growth factor-binding protein-1 (IGFBP-1) binds to insulin-like growth factors (IGFs) and has been shown to inhibit or stimulate cellular responses to IGF-I *in vitro*. This capacity of IGFBP-1 to inhibit or stimulate IGF-I actions correlates with its ability to form stable high molecular weight multimers. Since the ability of some proteins to polymerize is dependent upon transglutamination, we determined if tissue transglutaminase could catalyze this reaction and the effect of polymerization of IGFBP-1 upon IGF-I action. Following incubation with pure tissue transglutaminase (Tg), IGFBP-1 formed covalently linked multimers that were stable during SDS-polyacrylamide gel electrophoresis using reducing conditions. Dephosphorylated IGFBP-1 polymerized more rapidly and to a greater extent compared with native (phosphorylated) IGFBP-1. Exposure to IGF-I stimulated transglutamination of IGFBP-1 *in vitro*. An IGFBP-1 mutant in which $Gln^{66}$-$Gln^{67}$ had been altered to $Ala^{66}$-$Ala^{67}$ (Q66A/Q67A) was relatively resistant to polymerization by Tg compared with native IGFBP-1. Tg localized in fibroblast membranes was also shown to catalyze the formation of native IGFBP-1 multimers, however, Q66A/Q67A IGFBP-1 failed to polymerize. Although the mutant IGFBP-1 potently inhibited IGF-I stimulated protein synthesis in pSMC cultures, the same concentration of native IGFBP-1 had no inhibitory effect. The addition of higher concentrations of native IGFBP-1 did inhibit the protein synthesis response, and this degree of inhibition correlated with the amount of monomeric IGFBP-1 that was present. In conclusion, IGFBP-1 is a substrate for tissue transglutaminase and Tg leads to the formation of high molecular weight covalently linked multimers. Polymerization is an important post-translational modification of IGFBP-1 that regulates cellular responses to IGF-I.**

Insulin-like growth factors (IGF-I and IGF-II)[1] are associated with their specific binding proteins (IGFBPs) in extracellular fluids, and these binding proteins regulate IGF actions (1, 2). The mechanisms responsible for IGFBP-induced changes are not well defined. Several post-translational modifications have been shown to alter the affinity of IGFBPs for IGFs, including phosphorylation (3, 4), proteolysis (5, 6), and polymerization (7, 8). Dephosphorylation of human IGFBP-1 lowers its affinity for IGF-I by 6-fold (9), and the addition of dephosphorylated IGFBP-1 to cells in culture has been shown to enhance the capacity of IGF-I to stimulate DNA synthesis (1, 10). In contrast, phosphorylated IGFBP-1 inhibits IGF-I actions (2, 10). Our laboratory has previously demonstrated that polymerized forms of IGFBP-1 were present in human amniotic fluid and were abundant in the chromatographic fractions of IGFBP-1 purified from amniotic fluid that had the capacity to potentiate IGF-I-stimulated thymidine incorporation into porcine smooth muscle cells (pSMC) (1, 7, 10). In contrast, chromatographic fractions of purified IGFBP-1 that inhibited IGF-I actions had no detectable multimers (10). The mechanism(s) accounting for the appearance of these multimers have not been determined.

Tissue transglutaminase (transglutaminase type II) is a calcium-dependent enzyme that catalyzes the formation of isopeptide cross-links between glutamine and lysine residues and also can attach primary amines to peptide-bonded glutamines. The isopeptide cross-links are stable and resistant to proteolysis, thereby increasing resistance to chemical, enzymatic, or mechanical disruption. Tissue transglutaminase (Tg) activity is widely distributed in many tissues and organs, and it has been localized to the cytoplasm (11), cell surface (12), and extracellular matrix (13). The ability of some proteins to polymerize and form covalently linked multimers is dependent upon this activity (14, 15). Therefore, we speculated that IGFBP-1 might be a substrate for tissue transglutaminase and that polymerization of IGFBP-1 might change its ability to modulate IGF-I actions *in vitro*. The current studies were undertaken to determine whether tissue transglutaminase could catalyze the polymerization of IGFBP-1, to identify the factors that enhanced or inhibited polymerization, and to analyze the effect of polymerization upon IGF-I actions.

## EXPERIMENTAL PROCEDURES

*Materials*—pSMC were obtained from aortic explants and maintained in our laboratory as previously described (16). Human fetal fibroblasts (GM 10) were purchased from the Human Mutant Genetic Cell Repository (Camden, NJ). Calf serum was purchased from Colorado Laboratories Inc. (Logan, UT). [$^{35}$S]Methionine was from ICN

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‡ To whom correspondence should be addressed: Dept. of Medicine, CB7170, University of North Carolina, School of Medicine, Chapel Hill, NC 27599. Tel.: 919-966-4735; Fax: 919-966-6025; E-mail: endo@med.unc.edu.

[1] The abbreviations used are: IGF, insulin-like growth factor; IGFBP-1, insulin-like growth factor-binding protein; Tg, tissue transglutaminase; pSMC, porcine smooth muscle cells; CHO, Chinese hamster ovary; EMEM, Eagle's minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

Biomedical Inc. (Costa Mesa, CA). Tissue culture media, fetal calf serum, penicillin, streptomycin, and Geneticin (G418) were from Life Technologies, Inc. IGF-I was a gift from Genentech (South San Francisco, CA). Tissue transglutaminase and cystamine were purchased from Sigma. Native IGFBP-1 was obtained from media conditioned by CHO cells that had been transfected with an expression plasmid that contained the human IGFBP-1 cDNA (CHOBP1-D6) as previously described (3). Polyclonal rabbit antiserum for human IGFBP-1 was prepared as previously described (17). A monoclonal antibody to tissue transglutaminase was purchased from NeoMarker (Fremont, CA). Polyvinylidene difluoride transfer membranes were obtained from Millipore Corp. (Bedford, MA).

*Tissue Culture*—Human fetal fibroblast (GM 10) cells were maintained in Eagle's minimum essential medium (EMEM) (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) calf serum, penicillin (100 units/ml), and streptomycin (100 $\mu$g/ml). pSMC were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 $\mu$g/ml streptomycin). The medium was changed on the third day after plating, and the cultures reached 80% confluency in another 3 days, at which time they were used for experiments. GM-10 cells were used between passages 8 and 16, and pSMC were used between passages 4 and 8.

*Polymerization of IGFBP-1 with Tissue Transglutaminase*—IGFBP-1 (50 ng) was incubated with 0.5 microunits of pure tissue transglutaminase (purified from guinea pig liver) in reaction buffer containing 100 mM Tris-HCl, 20 mM dithiothreitol, 2.5 mM $CaCl_2$, 10% (v/v) glycerol, pH 8.5, for the indicated times at 37 °C (final assay volume 30 $\mu$l). The reactions were terminated by the addition of 4× Laemmli buffer containing 400 mM dithiothreitol and boiling for 10 min. The proteins were analyzed by 10% SDS-PAGE with immunoblotting for IGFBP-1 (7–9). The immune complexes were visualized using an alkaline phosphatase-conjugated anti-rabbit IgG and a phosphatase-dependent color development system as previously described (18). For Western ligand blotting, the proteins were transferred to polyvinylidene difluoride membranes; probed in 4.0 ml of 10 mM Tris-HCl, 150 mM NaCl, 0.05% sodium azide, 1% bovine serum albumin (pH 7.4) containing $^{125}$I-IGF-I (100,000 cpm/ml), specific activity 125 $\mu$Ci/$\mu$g; and then washed and visualized by autoradiography as described previously (19). Densitometric quantification of the bands was performed by scanning the film and analyzing the band intensities using NIH Image.

*Polymerization of IGFBP-1 in Human Fetal Fibroblast in Cultures*—GM 10 cells were grown to 80% confluency on 24-well culture plates (Falcon Labware, division of Becton Dickinson, Franklin, NJ; catalog no. 3047). The cultures were rinsed once with serum-free EMEM and incubated for 5 h in 250 $\mu$l of same medium. After the incubation, medium was replaced with serum-free EMEM containing 1 $\mu$g/ml of either native or dephosphorylated IGFBP-1 and incubated for 20–40 min at 37 °C, and then 30 $\mu$l of each sample was collected. The reactions were terminated by the addition of 4× Laemmli buffer containing 400 mM dithiothreitol. The reaction products were analyzed by SDS-PAGE with Western immunoblotting for IGFBP-1.

*Preparation of Membrane Extract from Human Fibroblasts*—GM 10 cells were grown to confluency on 10-cm tissue culture dishes (Falcon; catalog no. 3003). The cultures were rinsed with serum-free EMEM and incubated overnight in the same medium in the presence or absence of IGF-I (30 ng/ml). The cells were scraped from the dishes with a cell scraper and washed three times with ice-cold $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline. The washed cells were sonicated in sonication buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM KCl, 0.3 mM $Na_2HPO4$, 1 mM $NaHCO_3$, 250 mM sucrose, 1 mM phenylmethanesulfonyl fluoride, 1 $\mu$g/ml pepstatin, 1 $\mu$g/ml leupeptin). The cell lysate was centrifuged at 16,000 × *g* for 15 min, and the pellet was washed with the sonication buffer twice, solubilized in the same buffer containing 10 mM CHAPS, and centrifuged at 16,000 × *g* for 15 min. The transglutaminase activity in the detergent-soluble membrane extracts was determined by adding 10.5 $\mu$g of membrane protein to 30 $\mu$l of the assay buffer described previously except that the tubes also contained 25 ng of IGFBP-1. To determine whether polymerization was being catalyzed by Tg, some tubes contained cystamine (20 mM), a specific inhibitor of Tg activity (20).

*Plasmid Construction for Expression of the IGFBP-1 Mutant*—A full-length human IGFBP-1 cDNA was cloned into the *Hin*dIII and *Xba*I sites of a mammalian expression vector pRcRSV (pRcRSV-hBP-1) that was prepared from the plasmid pRcCMV (Invitrogen, La Jolla, CA). The pRcRSV-hBP-1 contains a bacteriophage origin of replication that allows production of plasmid DNA in a single-stranded form suitable for site-directed mutagenesis. The mutant IGFBP-1 cDNA was generated using site-directed mutagenesis (21). Single-stranded phagemid DNA was generated from pRcRSV-hBP-1, and the substitutions were introduced using synthetic oligonucleotide as a substrate for antisense DNA synthesis. The sequence of complementary oligonucleotide was 3′-CGT-GCAGAGGTGCCGCCTCCCCCGGCA-5′. This encoded conversions of $Gln^{66}$ to Ala and $Gln^{67}$ to Ala (designated pRcRSV-hBP-1Q66A/Q67A). The cDNA was sequenced, and the clones containing correct sequence were amplified. The plasmid DNA was purified using silica gel anion exchange resin chromatography (Qiagen, Chatsworth, MA).

*Transfection of Chinese Hamster Ovary Cells*—CHO-K1 cells were obtained from Lineberger Comprehensive Center Tissue Culture Facility (University of North Carolina). Cells were maintained in $\alpha$-modified Eagle's minimum essential medium containing 10% fetal calf serum, penicillin, and streptomycin. CHO cells were transfected with the pRcRSV-hBP-1Q66A/Q67A using a standard calcium phosphate precipitation procedure (22). The positive clones were selected with 800 $\mu$g/ml of G418 and maintained in a long term culture in 400 $\mu$g/ml G418.

*Protein Purification*—The Q66A/Q67A mutant IGFBP-1 was purified from conditioned medium of the pRcRSVhBP-1Q66A/Q67A-transfected CHO cells using procedures identical to those used to purify native IGFBP-1 as described previously (3). Twenty micrograms of native IGFBP-1 was dephosphorylated by incubating it with 50 units of calf intestinal alkaline phosphatase at 37 °C in 50 mM Tris-HCl, 0.1 mM EDTA (pH 8.5) for 16 h and repurified by reverse phase high pressure liquid chromatography. The purity of each form of IGFBP-1 was determined by SDS-PAGE with silver staining. Analysis of each form revealed a single monomeric band.

*[$^{35}$S]Methionine Incorporation into pSMC*—pSMC were grown to 80% confluency on 24-well culture plates. The cultures were rinsed once with serum-free DMEM and incubated overnight in the same media. After the incubation, media were replaced with 0.25 ml of methionine-free DMEM supplemented with 2 mM $CaCl_2$ for 4 h in the presence of 0.05 $\mu$Ci/well [$^{35}$S]methionine (specific activity, 1, 206 Ci/mmol), IGF-I (0 or 50 ng/ml), and various concentrations of either native or mutant IGFBP-1 (0–2,500 ng/ml). The plates were placed on ice, washed with ice-cold phosphate-buffered saline twice, and incubated with 10% trichloroacetic acid for 10 min. The trichloroacetic acid-precipitable radioactivity was solubilized in 1% SDS, 0.1 N NaOH and scintillation mixture (ScintiSafe™ Econo2; Fischer, Fair Lawn, NJ) and counted in a liquid scintillation counter (Beckman Instruments). Statistical comparisons were performed using a paired Student's *t* test.

## RESULTS

*IGFBP-1 Is a Substrate for Tissue Transglutaminase*—Following exposure to pure tissue Tg, IGFBP-1 formed covalent multimers that were stable during SDS-PAGE and were not altered with reducing agents (Fig. 1*A*). Incubation with tissue transglutaminase was associated with a time-dependent increase in dimer and multimer formation (Fig. 1*A*). Scanning densitometry showed that 57% of the total IGFBP-1 was present as polymerized forms after 5 h of incubation. When increasing concentrations of Tg were added, there was a progressive increase in dimer formation (Fig. 1*B*).

*Dephosphorylated IGFBP-1 Polymerizes More Readily Compared with Native IGFBP-1*—To determine whether the phosphorylation status of IGFBP-1 affects its ability to polymerize, dephosphorylated IGFBP-1 (50 ng) was incubated with increasing concentrations of tissue Tg (0–1.0 microunits/tube) for 30 min at 37 °C, and the products of the reaction were analyzed by SDS-PAGE using reduced conditions (Fig. 2). Dephosphorylated IGFBP-1 polymerized after exposure to 0.1 microunits of tissue Tg, whereas native IGFBP-1 required 0.4 microunits (Fig. 1*B*) or 0.5 microunits (Fig. 2) of Tg to detect polymerization. Dephosphorylated IGFBP-1 polymerized to a greater extent with each concentration of tissue Tg that was used. Therefore, dephosphorylation of IGFBP-1 enhances its susceptibility to Tg-catalyzed polymerization *in vitro*.

*Alteration of Residues $Gln^{66}$ and $Gln^{67}$ in IGFBP-1 Inhibits Its Polymerization by Tissue Transglutaminase*—To determine whether the adjacent glutamine residues, $Gln^{66}$ and $Gln^{67}$, were involved in the formation of Tg catalyzed polymerization, the Q66A/Q67A IGFBP-1 mutant was incubated with various concentrations of tissue Tg for 30 min at 37 °C, and the prod-

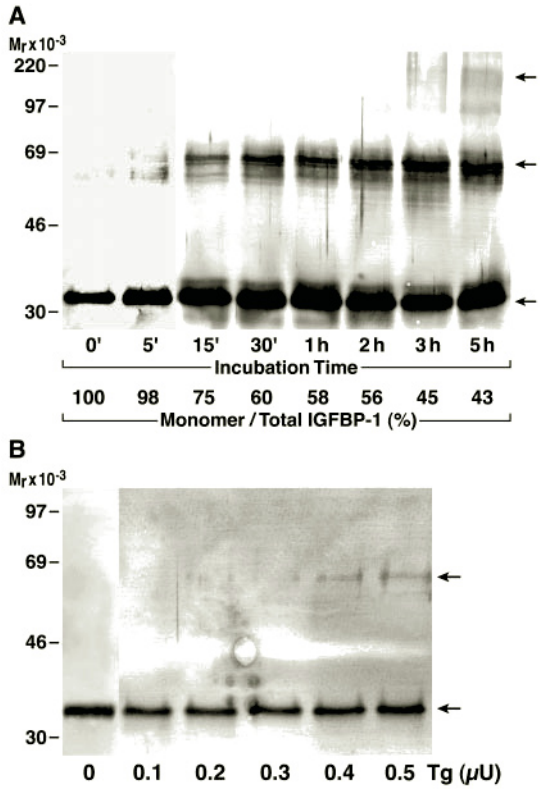


FIG. 1. **Polymerization of IGFBP-1 with tissue transglutaminase**. Native IGFBP-1 (50 ng) was incubated with 0.5 microunits (*μU*)/tube of tissue transglutaminase for the indicated time periods (*A*) or with increasing concentrations (0–0.5 microunits/tube) of tissue transglutaminase for 30 min (*B*) at 37 °C. The reaction products were analyzed by 10% SDS-PAGE followed by immunoblotting using human IGFBP-1 antiserum. The *arrows* denote monomer, dimer, and multimer forms of IGFBP-1. For *A*, the intensities of all of the bands were determined by scanning densitometry. The values shown represent the percentage of total scanning units that was detected as the IGFBP-1 monomer.

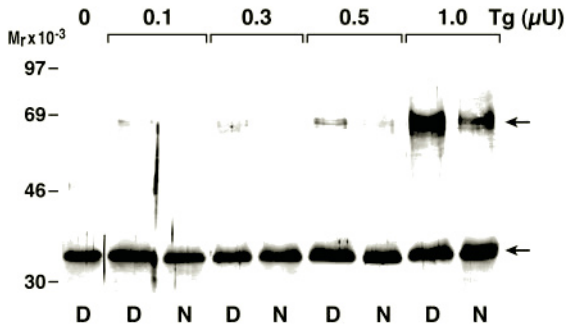


FIG. 2. **Polymerization of dephosphorylated IGFBP-1 with increasing concentrations of tissue transglutaminase.** Dephosphorylated IGFBP-1 was prepared as described under "Experimental Procedures." To determine whether its phosphorylation state would affect its polymerization, native IGFBP-1 (*N*) or dephosphorylated IGFBP-1 (*D*) (50 ng) was incubated with increasing concentrations (0.1–1.0 microunits (*μU*)/tube) of tissue transglutaminase for 30 min at 37 °C. The reaction products were analyzed by 10% SDS-PAGE with Western immunoblotting using human IGFBP-1 antiserum. The *bottom arrow* denotes the monomeric form, and the *top arrow* denotes the dimeric form of IGFBP-1.

ucts of the reaction were analyzed by SDS-PAGE using reducing conditions. The Q66A/Q67A mutant IGFBP-1 required 1.0 microunit of Tg to polymerize (Fig. 3), and only minimal amounts of dimer were detected. In contrast, the same concentration of native IGFBP-1 was polymerized to a much greater extent by 0.5 microunits of Tg. This reveals that substitution for $Gln^{66}$-$Gln^{67}$ in IGFBP-1 inhibits transglutamination and

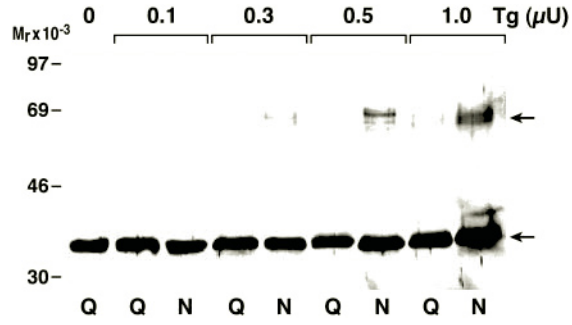


FIG. 3. **Polymerization of Q66A/Q67A mutant IGFBP-1 with increasing concentrations of tissue transglutaminase.** Native IGFBP-1 (*N*) or Q66A/Q67A mutant IGFBP-1 (*Q*) (50 ng) was incubated with increasing concentrations (0.1–1.0 microunits (*μU*)/tube) of tissue transglutaminase for 30 min at 37 °C. The reaction products were analyzed by 10% SDS-PAGE with Western immunoblotting using human IGFBP-1 antiserum. The *bottom arrow* denotes the monomeric form, and the *top arrow* denotes the dimeric form of IGFBP-1.

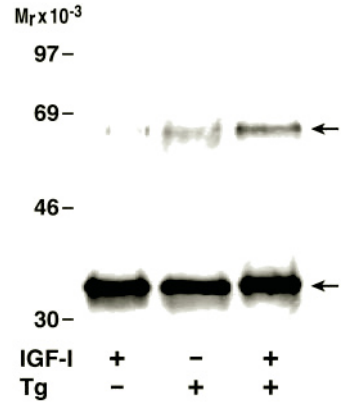


FIG. 4. **Enhancement of polymerization of IGFBP-1 by IGF-I.** Native IGFBP-1 (50 ng) was incubated with 1 ng of IGF-I for 1 h at room temperature followed by the addition of 0.3 microunits/tube of tissue transglutaminase, and the incubation was continued for 1 h at 37 °C. The reaction products were analyzed by 10% SDS-PAGE with Western immunoblotting using human IGFBP-1 antiserum. The *bottom arrow* denotes the monomeric form, and the *top arrow* denotes the dimeric form of IGFBP-1.

that $Gln^{66}$-$Gln^{67}$ is probably one of the cross-linking sites in IGFBP-1.

*Enhancement of Polymerization of IGFBP-1 by IGF-I*—Native IGFBP-1 was incubated with IGF-I at room temperature for 30 min. The IGF-I·IGFBP-1 complex was then incubated with 0.3 microunits of tissue Tg for 1 h at 37 °C, and the reaction products were analyzed by SDS-PAGE using reducing conditions (Fig. 4). The complexes containing IGF-I showed enhanced polymerization of IGFBP-1 by Tg compared with IGFBP-1 that was not exposed to IGF-I. Incubation with IGF-I and no Tg had no effect.

*Polymerization of IGFBP-1 by Exposure to Fetal Fibroblast Cultures*—Polymerization of IGFBP-1 had been observed previously in cell culture supernatants (7). Since tissue Tg is widely distributed in many tissues and organs, we determined if exposure of IGFBP-1 to fibroblast cultures would allow polymerization. Native and dephosphorylated IGFBP-1 were polymerized within 20 min, when IGFBP-1 (1 μg/ml) was added to GM 10 fetal fibroblast cultures (Fig. 5). Dephosphorylated IGFBP-1 was more readily polymerized compared with native IGFBP-1 by cell-associated Tg.

*Polymerization of IGFBP-1 Incubated with Fetal Fibroblast Membrane Extract*—Since tissue Tg is not secreted into the cell culture medium, we hypothesized that the polymerization of IGFBP-1 might be occurring on cell surfaces. To determine

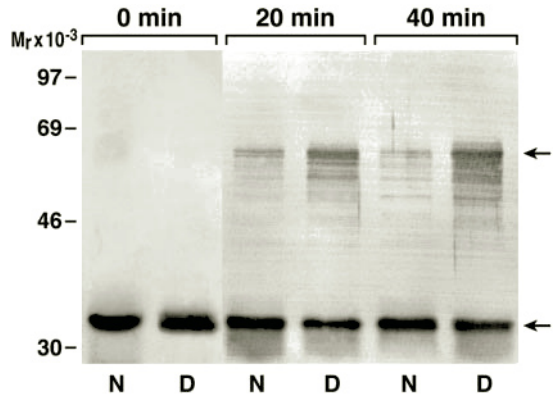


FIG. 5. **Polymerization of IGFBP-1 incubated with GM 10 fetal fibroblasts in culture.** GM 10 cells were grown to 80% confluency on 24-well tissue culture plates. Cells were incubated with serum-free EMEM containing either native (*N*) or dephosphorylated (*D*) IGFBP-1 (1 $\mu$g/ml) for the indicated times. Thirty microliters of each sample was collected and analyzed by SDS-PAGE with immunoblotting. The *bottom arrow* denotes the monomeric form, and the *top arrow* denotes the dimeric form of IGFBP-1.

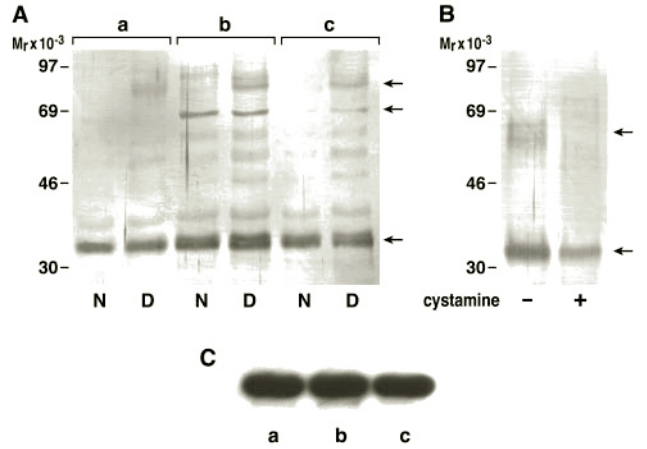


FIG. 6. **Polymerization of IGFBP-1 incubated with GM 10 fetal fibroblast membrane extracts.** GM 10 cells were grown to confluency on 10-cm tissue culture plates. In *A*, the cells were incubated with serum-free EMEM in the absence (*lanes a* and *b*) or presence (*lane c*) of IGF-I (30 ng/ml). After the incubation, the cellular membrane extracts were prepared as described under "Experimental Procedures." Each membrane extract was incubated with either native (*N*) or dephosphorylated IGFBP-1 (*D*) (25 ng) in the absence (*lanes a* and *c*) or presence (*lane b*) of IGF-I (1 ng/tube) for 14 h at 37 °C. The reactions were analyzed by SDS-PAGE with immunoblotting for IGFBP-1 (*A*). The *bottom arrow* denotes the monomeric form, and the *top two arrows* denote the dimeric and trimeric forms of IGFBP-1, respectively. *B*, the membrane extract was incubated with dephosphorylated IGFBP-1 (25 ng) in the presence and absence of cystamine (20 mM) for 6 h at 37 °C. The reactions were analyzed by SDS-PAGE with immunoblotting for IGFBP-1. *C*, the same membrane extracts were analyzed by SDS-PAGE and immunoblotted with anti-tissue transglutaminase antibody.

whether cellular membrane extracts could facilitate polymerization of IGFBP-1 and whether IGF-I altered the extent of polymerization, IGFBP-1 was incubated with fibroblast membrane extracts. Cell membrane extracts were prepared from cells that had been incubated for 14 h, and the samples were analyzed by SDS-PAGE under the reducing conditions (Fig. 6*A*). Exposure of the cultures to IGF-I enhanced polymerization of both phosphorylated and dephosphorylated IGFBP-1 compared with membrane extracts from control cultures exposed only to serum-free medium. However, if IGF-I was added *in vitro* directly to the membrane extract, the extent of polymerization was much greater. When cystamine, a specific inhibitor of Tg, was coincubated with IGFBP-1 and the membrane extracts, no polymerization was observed (Fig. 6*B*). Fig. 6*C* shows that these cellular membrane extracts contained equivalent concentrations of Tg.

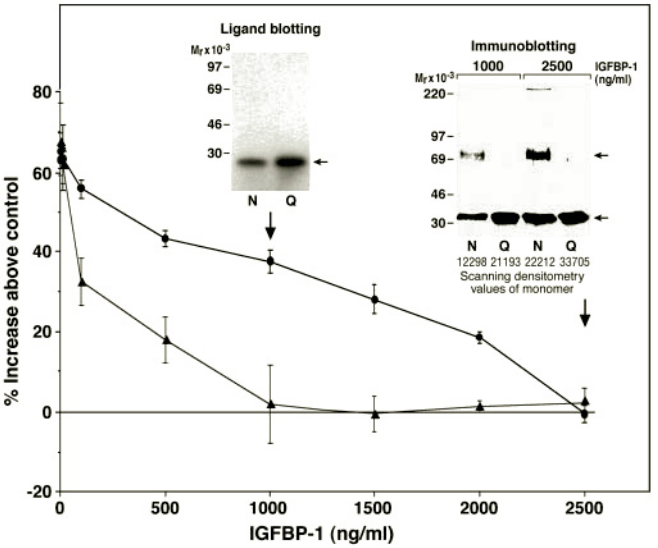


FIG. 7. **[$^{35}$S]methionine incorporation into pSMC protein synthesis was measured using [$^{35}$S]methionine incorporation into protein as described under "Experimental Procedures."** pSMC were grown to 80% confluency on 24-well tissue culture plates. The cultures were incubated with serum-free DMEM for overnight, and then media were replaced with methionine-free DMEM containing 0 or 50 ng/ml of IGF-I, different concentrations of either native (●) or Q66A/Q67A mutant IGFBP-1 (▲), and [$^{35}$S]methionine for 4 h. The amount of [$^{35}$S]methionine activity incorporated into cellular protein was measured. The results are expressed as a percentage of increase over control cultures that were incubated with methionine-free DMEM without IGF-I or IGFBP-1. Each value is mean ± S.E. of triplicate determinations. The *inset* shows a Western ligand blot and immunoblot of the media samples obtained at the end of the incubation from the cultures exposed to 20 $\mu$l of native or Q66A/Q67A IGFBP-1. The scanning units that were detected in the monomeric band (*lower arrow*) are shown.

*Q66A/Q67A Mutant IGFBP-1 Inhibits IGF-I-stimulated Protein Synthesis on pSMC in Culture*—pSMC were grown to 80% confluency in 24-well culture plates and the ability of IGF to stimulate [$^{35}$S]methionine incorporation into protein was determined. IGF-I stimulated the incorporation of [$^{35}$S]methionine into pSMC by 60% above the basal level. The addition of native IGFBP-1 at concentrations as high as 1,000 ng/ml reduced this response by 20%, although the decrease was not significant ($p$ = NS). Native IGFBP-1 (2,500 ng/ml) was required to obtain complete inhibition. In contrast, the addition of 1,000 ng/ml Q66A/Q67A mutant IGFBP-1 resulted in complete inhibition of protein synthesis that had been stimulated by IGF-I ($p < 0.001$) (Fig. 7). Analysis of the forms of IGFBP-1 that were present in the media at the end of the incubation showed that the native IGFBP-1 polymerized, but the Q66A/Q67A mutant IGFBP-1 was present only in the monomeric form. The scanning densitometric values of 1,000 ng/ml of mutant IGFBP-1 and 2500 ng/ml of native IGFBP-1 showed that approximately the same amount of monomeric IGFBP-1 was present. When the polyvinylidene difluoride membrane was probed with radiolabeled IGF-I, the intensity of the native IGFBP-1 monomeric band was reduced compared with Q66A/Q76A mutant IGFBP-1 monomer. More importantly, the native IGFBP-1 polymeric form did not bind to radiolabeled IGF-I, and no band could be detected.

## DISCUSSION

The current studies demonstrate that IGFBP-1 is a substrate for tissue Tg and that Gln$^{66}$-Gln$^{67}$ is a one of the amine acceptor sites in IGFBP-1. This polymerization-resistant mutant

| | -12 | | | | | | | | | | | | * | * | | | | | | | | | | +12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| γ-fibrin | I | I | P | F | N | **R** | L | T | I | G | **E** | G | Q | Q | **H** | **H** | L | G | G | A | **K** | Q | A | G | **D** | V |
| Fibronectin | | | | | | | | | | NH2- | **E** | A | Q | Q | I | V | Q | P | Q | S | P | L | T | V | S | Q |
| β A3-crystallin | | | | | | | NH2- | **E** | T | Q | T | V | Q | Q | **E** | L | **E** | S | L | P | T | T | **K** | M | A | Q |
| Plasminogen activator inhibitor-2 | M | T | P | **E** | N | F | T | S | C | G | F | M | Q | Q | I | Q | **K** | G | S | Y | P | **D** | A | I | L | Q |
| Osteonectin | | | | | | | | | | NH2- | A | P | Q | Q | **E** | A | L | P | **D** | **E** | T | **E** | V | V | **E** | **E** |
| IGFBP-1 | A | **R** | G | L | S | C | **R** | A | L | P | G | **E** | Q | Q | P | L | **H** | A | L | T | **R** | G | Q | G | A | C |

FIG. 8. **Comparison of amino acid sequences flanking the two adjacent glutamine residues that have been identified as amine acceptor sites.** Two adjacent glutamine residues (amine acceptor site) are *underlined*, and charged residues are in *boldface type* for IGFBP-1 and other proteins that have been noted to undergo transglutamination.

form of IGFBP-1 potently inhibits IGF-I-stimulated protein synthesis into pSMC. In contrast, when polymerizable native IGFBP-1 was added, much higher concentrations were required to inhibit IGF-I action. The polymerization of IGFBP-1 by tissue Tg was also shown to be altered by the phosphorylation state of IGFBP-1 and by the presence of IGF-I in the incubation buffer.

Numerous proteins have been demonstrated as substrates for Tg; however, a general consensus recognition sequence for transglutaminase has not been determined. Two directly adjacent glutamine residues have frequently been identified as amine-accepting sites. The γ-chain of fibrinogen (23) and fibronectin (24), which contain adjacent glutaminase residues, are substrates for factor XIIIa. Plasminogen activator inhibitor-2 (25), β A3-crystallin (26), and BM-40 (osteonectin) (27) are substrates for tissue Tg, and they have two directly adjacent glutamine residues that have been identified as amine acceptor sites. In addition, positively or negatively charged amino acid residues are often found near amine acceptor sites in the sequence of these proteins. Analysis of the sequences following $Gln^{66}$-$Gln^{67}$ in IGFBP-1 showed that both positively ($Glu^{65}$) and negatively ($His^{70}$) charged residues are present (Fig. 8). The positions of charged residues in several other substrates for tissue Tg are shown for comparison. Because of these similarities, we speculated that the adjacent glutamine residues in IGFBP-1 ($Gln^{66}$-$Gln^{67}$) could be amine acceptor sites. The Q66A/Q67A mutant form of IGFBP-1 that was generated was resistant to polymerization by pure tissue Tg and following exposure to cell cultures that contained tissue Tg in their membrane extracts. These findings strongly support the conclusion that tissue Tg is using these residues as amine acceptor sites for cross-linking in IGFBP-1.

IGFBP-1, -2, -3, and -5 have been shown to stimulate or inhibit IGF-I actions *in vitro* (28). Most studies have shown that excess concentrations of IGFBP-1 inhibit the IGF-I actions *in vitro*, particularly if high concentrations of IGFBP-1 are added using serum-free conditions (2, 10, 29–31). When platelet-poor plasma is added with IGF-I and dephosphorylated IGFBP-1, IGFBP-1 has been shown to potentiate the IGF-I-stimulated DNA synthesis in cultured fibroblasts and pSMC (1). The phosphorylation state of IGFBP-1 was also shown to be an important determinant of this response, and dephosphorylated IGFBP-1 was much more effective than phosphorylated IGFBP-1 (1, 9, 32). Analysis of the cell culture supernatants at the end of these incubations showed that the added IGFBP-1 had polymerized. Therefore, the current studies were undertaken to determine whether IGFBP-1 polymerized as a result of exposure to transglutaminase, if its phosphorylation status of IGF-I affected its capacity to polymerize, and if polymerization would be associated with a decrease in the ability to inhibit IGF-I-stimulated protein synthesis.

IGFBP-1 is a phosphoprotein that undergoes serine phosphorylation (9). Several phosphoisoforms of IGFBP-1 have been detected in serum, and the presence of highly phosphorylated forms of IGFBP-1 in physiologic fluids has been associated with conditions in which the target tissue actions of IGF-I are being inhibited (32, 33). Separation of phosphoisoforms of IGFBP-1 by anion exchange chromatography has shown that chromatographic fractions that contain phosphorylated forms inhibit IGF-I actions, whereas those that are enriched in nonphosphorylated forms did not inhibit IGF-I actions (9, 10, 28). In this study, dephosphorylated IGFBP-1 polymerized to a much greater extent compared with phosphorylated IGFBP-1. Since the ability of IGFBP-1 to polymerize has also been shown to be related to the loss of its capacity to inhibit IGF-I actions, these results suggest that this may be one mechanism by which phosphorylation of IGFBP-1 may enhance its capacity to inhibit IGF-I actions.

The results of several studies have shown that the loss of the ability of IGFBP-1, IGFBP-3, and IGFBP-5 to inhibit IGF-I actions is associated with a reduction in their affinities for IGF-I (9, 34–38). IGFBPs have high affinity for IGF-I, and their affinities are greater than that of the type-I IGF receptor. Therefore, factors such as dephosphorylation (3) or adherence to cell surfaces (18), which lower their affinity for IGF-I, will reduce their capacity to inhibit IGF-I actions, and in some cases this has been associated with stimulation of IGF-I actions (1). Our results showed that the multimeric form of IGFBP-1 (Fig. 7) that occurred following transglutamination had very low binding capacity for IGF-I and that polymerization was associated with a loss of IGFBP-1's inhibitory effect. Immunoblotting analysis at the end of incubation revealed that the concentration of monomeric IGFBP-1 correlated with the extent of inhibition of IGF-I action. This result suggests that polymerization is also an important mechanism by which the inhibitory effect of IGFBP-1 is attenuated.

The N-terminal domain of IGFBP-3 and -5 contain a binding site for IGF-I (39). In IGFBP-5, the IGF-I binding site is contained within amino acids 49–74, and in IGFBP-3 it is between residues 50 and 75. For IGFBP-5, the charged residue $Lys^{68}$ and the hydrophobic residues $Pro^{69}$, $Leu^{70}$, $Leu^{73}$, and $Leu^{74}$ are required for binding. In the IGFBP-1, residues $Pro^{68}$, $Leu^{69}$, and $Leu^{70}$ are homologous, suggesting that this is also an important site in IGFBP-1 for IGF-I binding. Our results demonstrate that following transglutamination, which occurs through $Gln^{66}$-$Gln^{67}$, the affinity of IGFBP-1 is markedly reduced. This suggests that cross-linking through these sites alters the conformation of the adjacent IGF-I binding site such that IGF-I has reduced access.

Our results also show that IGF-I stimulates the polymerization of IGFBP-1 by pure tissue Tg and by the Tg that was contained in fibroblast membrane extracts. The effect is direct and does not require the presence of some other component. Since IGF-I is probably binding to the region of IGFBP-1 that contains the amine acceptor sites, its presence may induce a conformational change in IGFBP-1 that stabilizes the conformation of the multimeric forms and facilitates cross-linking by tissue Tg.

Recently, tissue Tg has been shown to associate with $\beta_1$ and $\beta_3$ integrins, and it has been estimated that 5–40% of $\beta_1$ integrins on the cell surface are complexed with tissue transglutaminase. Tissue Tg on the cell surface has been shown to

increase cell adhesion and spreading on fibronectin (40). IGFBP-1 has been shown to bind to the $\alpha_5\beta_1$ integrin and stimulate cell migration independently of IGF-I (41). Ligand occupancy of $\alpha_5\beta_1$ by IGFBP-1 has been shown to induce focal adhesion kinase and PI 3-kinase. Therefore, IGFBP-1 could form an IGFBP-1-tissue Tg complex with the $\beta_1$ integrin, and transglutamination of IGFBP-1 could potentially modulate the $\alpha_5\beta_1$ integrin-linked signaling. Elucidation of the interaction between IGFBP-1 and tissue Tg will be a great help in further analysis of the biological significance of multiple IGFBP-1/IGF-I interactions that occur *in vivo*.

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